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13. SUPPLEMENTARY NOTES

14. ABSTRACT
The purpose of this work is to provide the research community with a robust functional screening approach for identification of novel oncogenic drivers of neuroblastoma as a starting point for the development of new therapies. Furthermore to use this technology to start identifying novel oncogenic drivers ourselves. To do this we have established a novel system to functionally screen candidate oncogenic drivers through the transformation of primary neural crest cells into neuroblastoma. Through work supported by this grant we have evidence that ARID1A is an important tumor suppressor that can collaborate with N-Myc in initiating neuroblastoma through our transformation of primary mouse neural crest cells into phenotypically accurate neuroblastoma. We have also advanced our screening technology by an over 300-fold improvement in the number of primary neural crest cells generated per isolated neural tube, which will allow us to screen highly complex pools of candidate neuroblastoma oncogenic drivers. We have also made significant advances in establishing complementary in vitro screening approaches that can validate positive hits from our tumor screen and that can identify oncogenic drivers that may be missed by our more stringent in vivo tumor assay.

15. SUBJECT TERMS
Neuroblastoma, Neural Crest, Oncogenes, Tumor suppressors, Functional Screen

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INTRODUCTION:

Neuroblastoma is responsible for approximately 15% of pediatric cancer deaths but the genes responsible for driving this malignancy are generally unknown. Other then N-Myc amplification the other common tumor suppressors and oncogenes are hidden within frequently recurrent large segmental chromosomal gains and losses that are found in high-risk neuroblastoma. This proposal is to develop tools and protocols to establish rapid functional sufficiency screens to help identify those hidden oncogenic drivers. Our screens will use primary mouse neural crest cells (NCC) the stem/progenitor cells that gives rise to the cell lineage responsible for neuroblastoma. In addition to establishing these news tools, in this proposal we are further using these new approaches to test candidate genes that are putative oncogenic drivers. We have prioritized for testing candidate tumor suppressors genes from the 1p36 chromosomal regions with particular focus on ARID1A and CHD5 since the loss of this region often associates with MYCN amplification. We will have also proposed screening candidate genes from the most common chromosomal gains and losses observe in high-risk neuroblastoma which in addition to 1p36LOH includes 11qLOH and 17q gain. The intent of this work is to start identifying novel oncogenic drivers as a starting point for the development of new therapies.

KEYWORDS: Neuroblastoma, Neural Crest Cells, Oncogenic Drivers, Genetic Screen, Tumor Suppressors, Oncogenes.

ACCOMPLISHMENTS:

Major Goals

1. **Identifying Tumor Suppressor of N-Myc amplification**
2. **Establishing a functional screen using neural crest cells**

Accomplished Under these goals

Major Task: Identifying Tumor Suppressor of N-Myc amplification

The pediatric cancer neuroblastoma (NBL) forms from immature cells of the sympathetic nervous system (SNS). A prominent category of high-risk NBL is MYCN amplification with loss of heterozygosity (LOH) at 1p36. This LOH includes the chromatin-remodeling factor, CHD5, a proposed tumor suppressor of NBL and can include ARID1A, a component of the SWI/SNF chromatin-remodeling complex. In the literature other tumors suppressor candidates have been proposed from this region, including CASZ1, CAMTA1, KIF1beta, mir-34a and RUNX3, which will be more the focus in our second major task.

Our first effort was to use data with the help of bioinformaticians at St. Jude Children's Research Hospital to mine unique data generated by the Pediatric Cancer Genome Project (PCGP) from whole genome sequencing of neuroblastoma tumors. The bioinformaticians also used publicly available NBL data from the TARGET genome project. The question we asked was based on work by Versteeg and others in the early 90's showed evidence of a proximal and distal tumor suppressor in the 1p36 locus with N-Myc amplified tumors always having a larger LOH. His hypothesis was that loss of that proximal tumor suppressor was necessary for N-Myc amplification and his group did cell fusion studies that supported that idea. He found the distal tumor suppressor was shared between both N-Myc single and N-

Myc amplified tumors and had a smallest region of overlap (SRO) in the 1p36.23-33 region. This is the tumor suppressor that the field has been trying to find by further refining the SRO in that region. There are multiple candidates proposed for the distal tumor suppressor with CHD5 being a lead candidate. However those 1p36 LOH studies were done without segregating N-Myc amplified from N-Myc single therefore not defining the SRO that is unique to N-Myc amplified NBL. For the proximal tumor suppressor Versteeg's group could only say that it is from 1p35-36.1 to the telomere suggesting that it is proximal to 1p36.33 and inclusive of 1p35-1p36.1. To my knowledge nothing else was accomplished towards finding that proximal tumor suppressor. However when Hogarty's paper showed mutations in ARID1A in neuroblastoma patients it was of interest because its location at 1p35-1p36.1. Shown in figure one we found that the SRO of all MYCN amplified NBL with 1p36LOH always included ARID1A (Figure 1).

A.

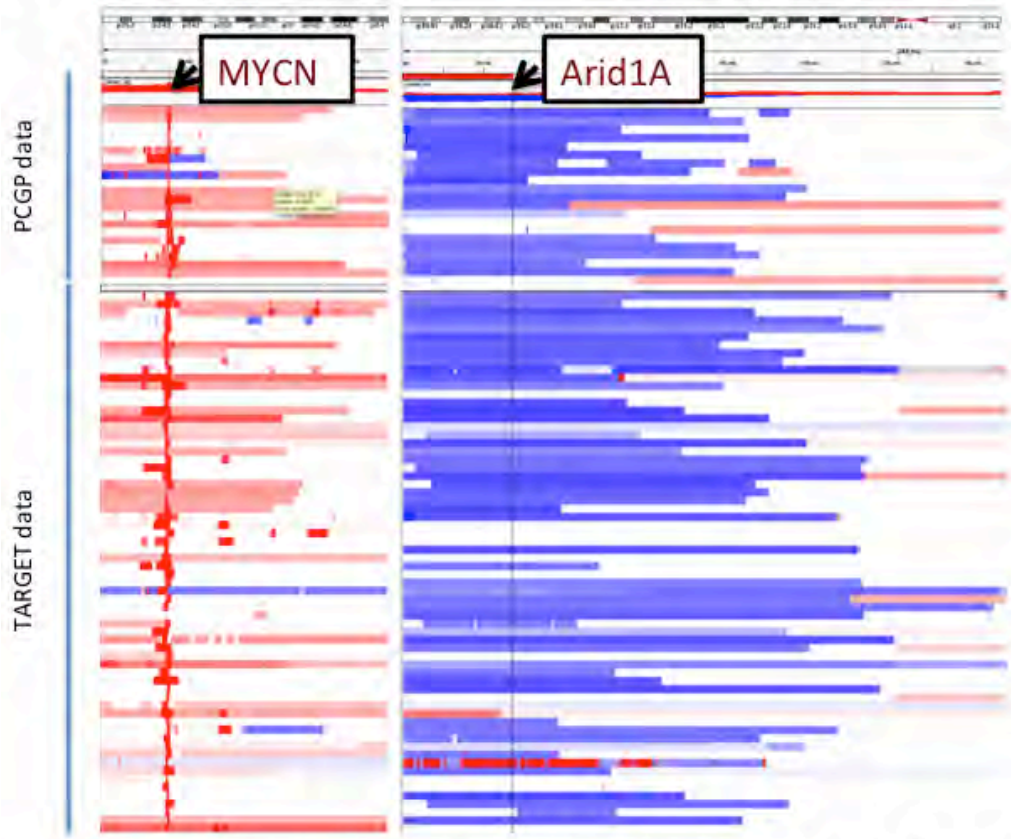

Figure 1: Loss of ARID1A correlates with MYCN gene amplification in whole genome sequencing data from the TARGET database and from the St. Jude Children's Research hospital Pediatric Cancer Genome Project (PCGP). A) Patient genomic data comparing the chromosomal region containing N-Myc and the 1p36 region shows with the vertical red lines indicating MYN amplification and the extent of chromosomal loss at th 1p36 locus indicated by the horizontal blue lines and all patients with MYCN amplification and 1p36LOH have loss of ARID1A.

Based on Versteeg's work and others and our confirmation of their work using up to date whole genome sequencing information we chose to target both ARID1A and CHD5 with N-Myc. Using our neural crest strategy loss of both tumor suppressors appeared to increase the transformation of primary NCCs by N-Myc from a 25% take rate to a 33% take rate (Figure 2A). Also loss of CHD5 failed to increase transformation by N-Myc and appear even to inhibit tumor take (Figure 2A). Additionally the tumors that were derived from these experiments were uniformly

positive for neuronal markers including TH and ASCL1, as well as Phox2B, a marker of undifferentiated NBL (Figure 2B). They also showed hallmarks of NBL with neuropil and ganglion cells, which is unique to this model and had not been observed in other mouse models of NBL by the reviewing pathologist. By electron microscopy tumors also showed dense core vesicles and microtubles forming axons. The pathologist designated these cancers as differentiating neuroblastoma subtype stromal poor. Overall this is supportive of a model that loss of ARID1A is the long

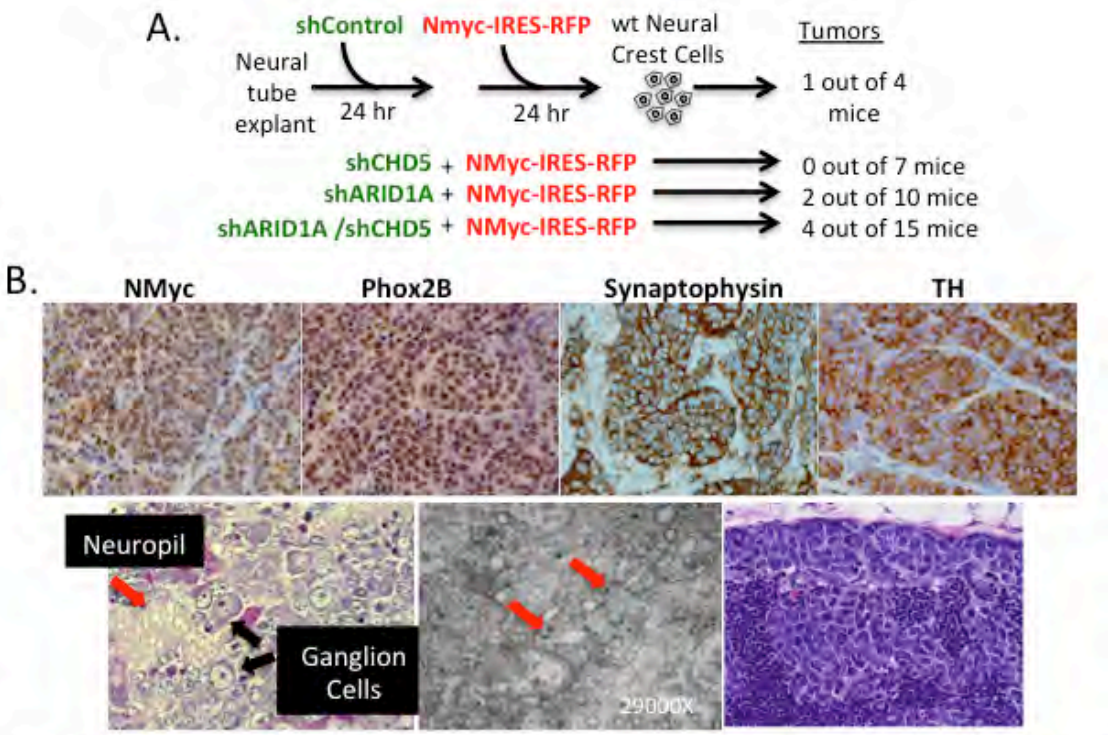

Figure 2: Transformation of Wild-type NCCs by NMyc and Knockdown of CHD5 and ARID1A Results In Neuroblastoma Tumors

A) E9.5 neural crest cells (NCCs) were isolated from wild-type embryos, infected with NMyc virus and shRNA to knockdown CHD5 and ARID1A, then injected into nude mice. Number of tumors per cohort of mice injected is given in right column.

B) NCC-derived tumors highly express neuronal genes (Phox2B, Synaptophysin, and TH, 40x magnification) and were classified as neuroblastoma, differentiating subtype, stroma poor. Tumors have neuropil (red arrow) and ganglion cells (black arrow) (60X) with transmission electron microscopy showing dense core vesicles (red arrows) (29000X). Tumors were confirmed by pathologist as neuroblastoma. Frequent metastases to lymph nodes was also observed.

sought after tumor suppressor in the 1p36 locus that allows cells to tolerate N-Myc amplification and that it cooperates with CHD5.

We also noted in primary NCCs (Figure 3A) and in cell lines derived from tumors generated by overexpression of N-Myc in p53 compromised NCCs (Figure 3B) that there was a positive correlation between N-Myc expression levels and ARID1A expression levels which might indicate that there is a regulatory loop with constitutive N-Myc expression causing increased expression of the tumor

suppressor ARID1A.

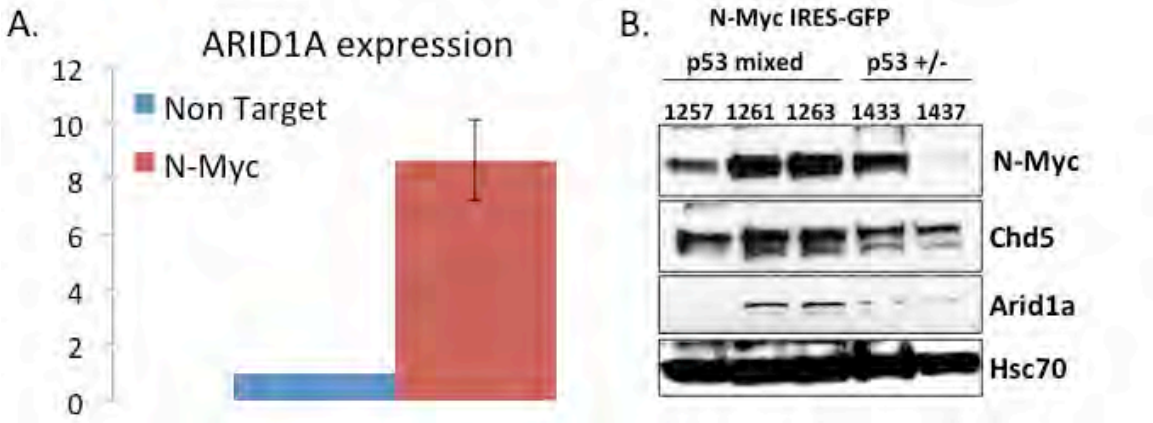

Figure 3: Overexpression of N-Myc correlates with increased expression of ARID1A. A) Primary neural crest cells were infected with N-Myc or non-Target control virus and ARID1A mRNA expression levels were quantified by QT-PCR. **B)** Cell lines derived from tumors from N-Myc transformed p53 compromised NCC show a correlation between ARID1A protein expression and N-Myc expression by western blot analysis.

We based our expression of N-Myc transgenes and shRNA constructs using MSCV promoters. This was done because it drives strong expression in neural tissue including neuroblastoma cell lines and in primary neural crest cells. We have determined that silencing of ARID1A either by using shRNA (Figure 4A and B) or C

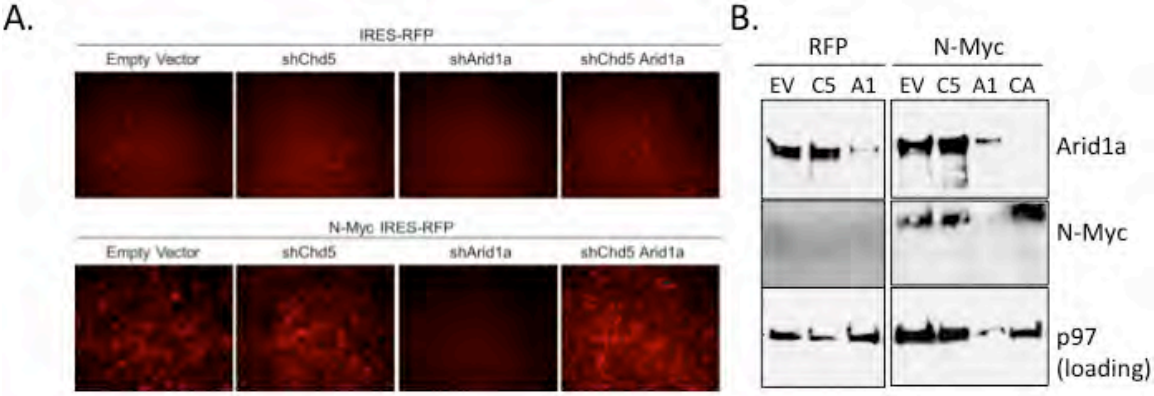

Figure 4: Silencing of ARID1A cause loss of expression from MSCV promoter that is reversed by loss of CHD5. Primary NCC infected with MSCV-N-Myc-IRES-RFP or MSCV-IRES-RFP show loss of expression from the MSCV promoter by immunofluoresence to RFP **(A)** or of N-Myc by expression **(B)** when ARID1A is silenced by shRNA. This is reversible if CHD5 is also silenced.

RISPR-Cas9 technology (Data not shown) leads to silencing of expression of N-Myc or of the red fluorescent protein (RFP) that is driven by the IRES downstream of the MSCV promoter in our constructs. We also speculate that there is a feedback loop leading to suppression of the shRNA itself as they are also driven off the MSCV promoter. Interestingly silencing of CHD5 in conjunction with silencing of ARID1A restores expression from the MSCV promoter (Figure 4A and B) showing a functional interaction of these two putative tumor suppressors from the 1p36 locus but also further confounding some of the results that we have generated to this point. This also explains issues that were arising in our inducible cell line (iN-Myc-AS and iN-Mys-FI) where we saw changing levels of ARID1A knockdown when we looked at expression of the gene during timecourse studies, proliferation studies and apoptosis studies. This necessitates us to rebuilding these systems, which we are currently doing (data not shown), but does not invalidate our results.

Now that we understand this technical issue we have been generating new reagents that are independent of the MSCV promoter. We have identified multiple shRNA driven off of the U6 promoter capable of silencing human ARID1A overexpressed in 293 cells (Figure 5A and B). We have also successfully identified multiple guide RNAs that can effectively target both mouse and human ARID1A that is also driven off the U6 promoter (Figure 5C). For expression of N-Myc we have cloned it downstream of multiple promoters and are testing to ensure that there is no

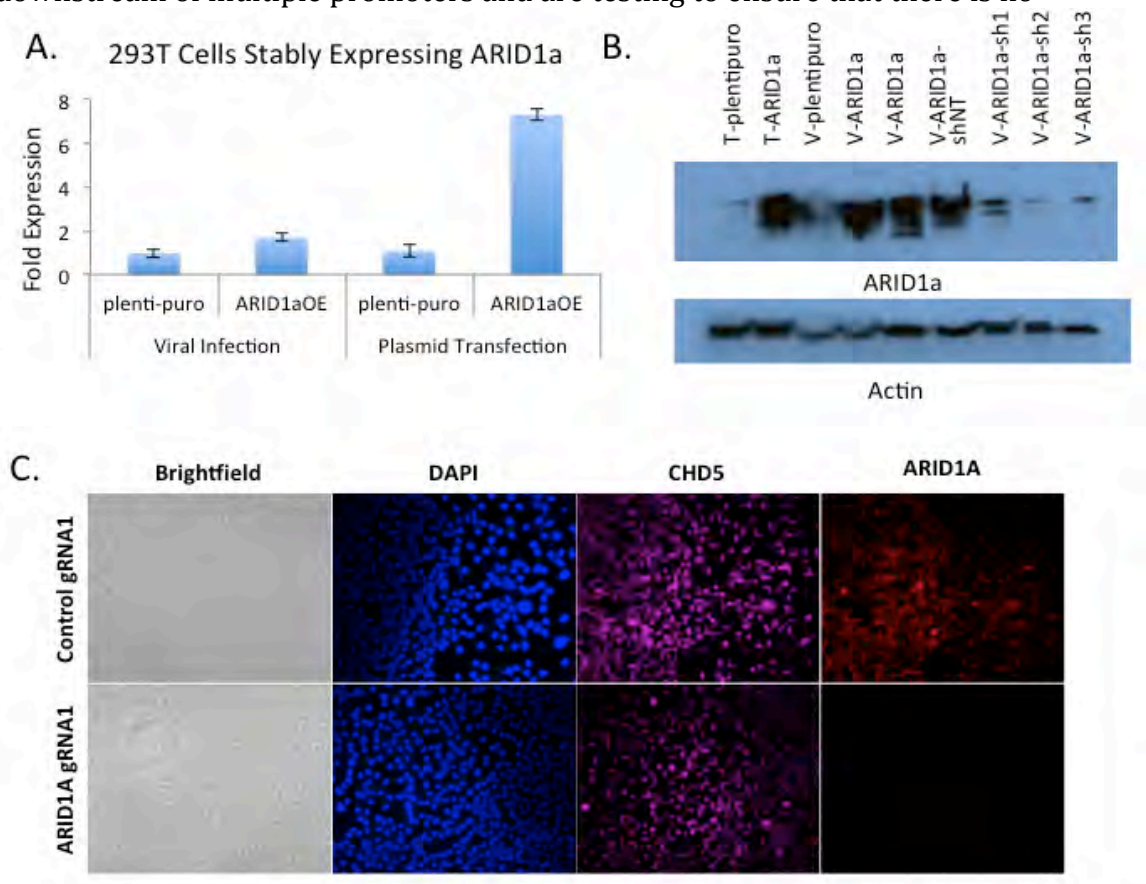

Figure 5: Gene silencing of ARID1A using alternate promoters and CRISPR-Cas9 technology. (A) Human ARID1A was overexpressed in 293 cells to generate target mRNA for testing of shRNAs. (B) All three shRNAs to ARID1A driven off U6 promoters silenced expression of human ARID1A. (C) Silencing of mouse ARID1A in primary neural crest cells using CRISPR-Cas9 nickase and ARID1A specific guide RNA shows loss of endogenous ARID1A expression by immunofluoresence.

silencing of its expression due to loss of ARID1A. We have also cloned N-Myc into a Piggybac transposon with N-Myc's own endogenous promoter since this transposon has a larger insert capacity than lentiviral vectors making it capable of carrying a region that encompasses promoter regulation that retains specific expression of the endogenous genes and the gene itself. Also transposon will insert tandomly into the genome mimicking N-Myc amplification.

Percentage of completion:
Subaim 1A: To further characterize the functional consequences of ARID1A loss in N-Myc induced NBL cell lines (ATCC)
60% completion

Subaim 1B. To assess the impact of N-Myc induction and loss of ARID1A on tumorigenesis in NBL cell lines.
80% completion

Subaim 1C. To assess if ARID1A is a haploinsufficient tumor suppressor in NBL
20% completion

Major Task: Establishing a functional screen using NCC

Neuroblastoma arises in the sympathoadrenal lineage of trunk NCCs and is the most common of the neural crest derived pediatric cancers representing 10% of all pediatric cancers and 15% of all pediatric cancer deaths. N-Myc amplification and segmental chromosomal changes with 17q gain, 1p loss of heterozygosity (LOH), 11q LOH and 3p LOH are the most frequent genetic alterations associated with high-risk NBL. Besides N-Myc, other common oncogenic drivers are currently unknown as the oncogenes and tumor suppressors from each of these segmental chromosomal gains and losses have not been defined. Thus, establishing the molecular etiology of this disease and finding tractable therapeutic targets are key challenges for neuroblastoma.

Though at the time of this grant proposal we had established a tractable system for transforming primary NCC, one major limitation we had was that our in vitro culturing of the NCC did not allow for expansion of cell numbers. This limited the potential complexity of the pools of candidates genes we would be capable of screening. We have tested multiple culturing conditions and identified a defined media that allows the long term culturing of these cells. While before we could only culture cells for a week and saw no expansion in cell numbers therefore only generating ~3,000 NCC per neural tube we can now grow the cells for at least a month at a time and generate $1x10^6$ cells per neural tube (Figure 6A). We have validated that these NCCs are being kept in an undifferentiated state while they are being expanded. They maintain markers of early neural crest, p75 and Sox 10 and markers of migratory neural crest, ASCL1/MASH1 by QT-PCR and immunofluorescence (Figure 6B and C). Additionally they lack marker of differentiation such as tyrosine hydroxylase (TH), which is prominently seen in sympathetic nervous tissue such as the adrenal gland (Figure 6D). Furthermore we verified that these cells still retain the capacity to differentiate as expected into neurons and Schwann cells once placed in neurogenic media becoming cells that expressing the neuronal markers MAP2 and Tuj1 as well as cells that express the Schwanian marker GFAP (Figure 6E). We have also established that we can take NCC with N-Myc overexpression in a p53 compromised background after expansion in chemically defined media and culturing in neurogenic media for 7 days and still see rapid subcutaneous tumor growth (Figure 7). Our advances in the tissue culturing of NCC will allow us to test much more complicated gene pools and to better optimize and validate each step of screens since we will have more material with which to work.

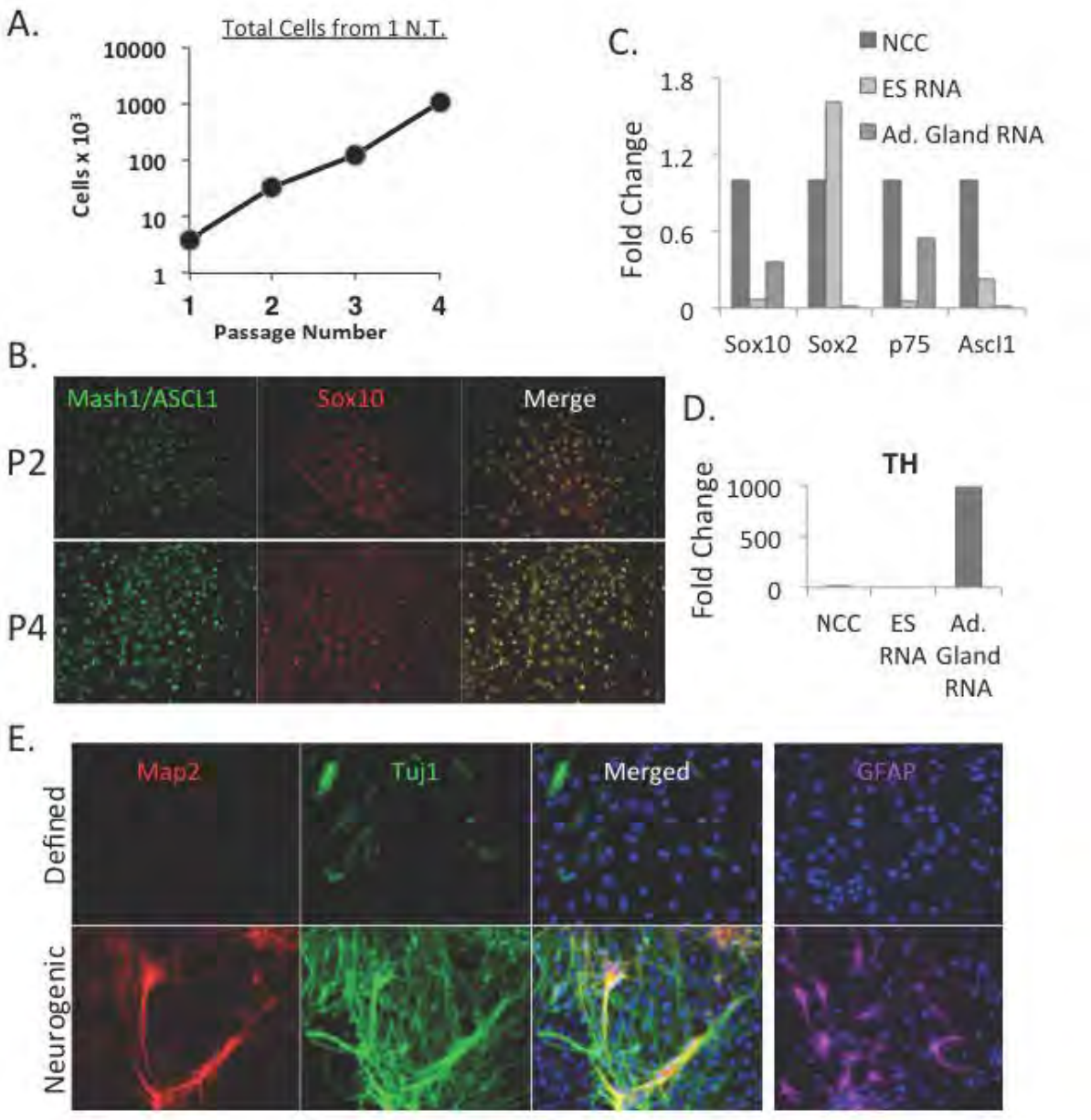

Figure 6: Establishment of long-term in vitro tissue culture growth of primary NCC
A) NCC grown in chemically defined media were enumerated weekly per neural tube. B) Both earlier passage cells (P2) or later passaged cells (P4) were positive for markers of early NCC by immunofluorescence. C) Later passage NCC were also positive for early neural crest markers by QT-PCR in comparison to mouse ES cells or adrenal gland. D) They also lacked a marker for more differentiated cells, tyrosine hydorxylase (TH). E) When placed in neurogenic media for 7 days, later passage NCC were still capable of generating differentiated neurons as seen by the the neuronal markers MAP2 and Tuj1 as well as Schwann cells as seen by the glial marker GFAP

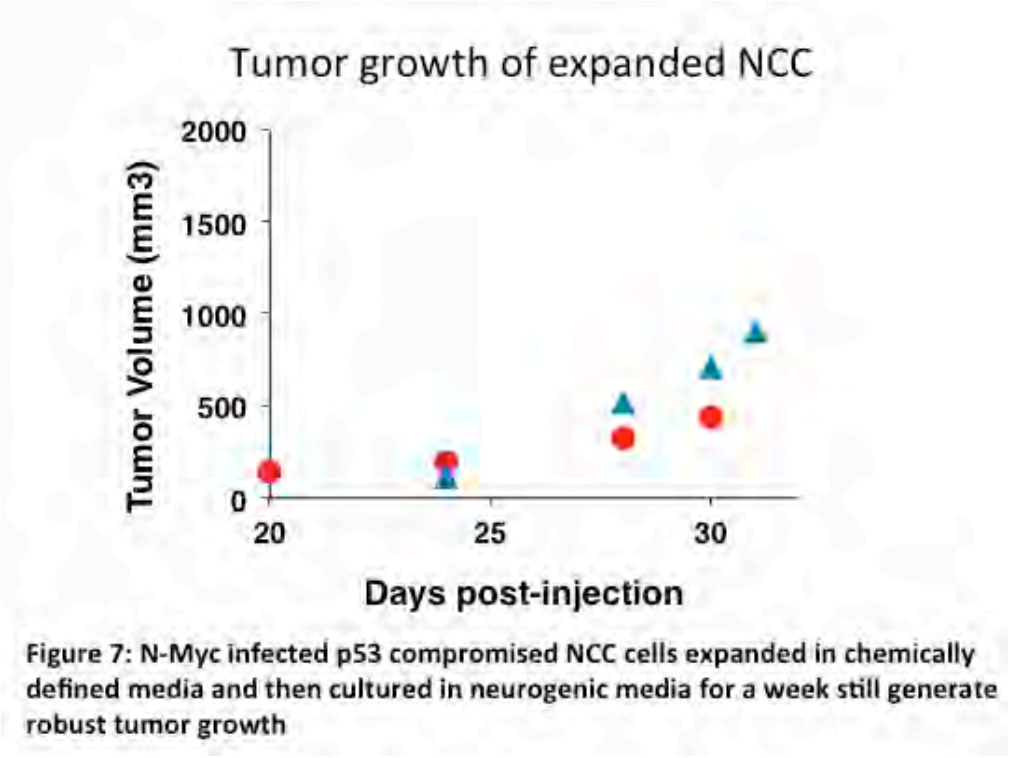

Figure 7: N-Myc infected p53 compromised NCC cells expanded in chemically defined media and then cultured in neurogenic media for a week still generate robust tumor growth

We also were interested in establishing in vitro screening system, which would allow us to have secondary approaches for screening candidate genes in addition to in vivo tumor screens. Also these screens would be less stringent that an in vivo tumor screen and so may allow us to expand the number of positive hits for oncogenic drivers that could later be verified in the more stringent in vivo screen. We have tested soft-agar colony assays, as this assay is considered to be the closest to in vivo tumor formation and also colony formation in low adherence plates since this has recently been validated to reproduce the effects of a soft agar colony assay but is a simpler protocol, which typically reduces experimental error (Figure 8 A-C)

To establish a screening library for 1p36 we used the newer but effective CRISPR-Cas 9 technology. Though we have vary promising data for ARID1A and CHD5 other genes from 1p36 LOH my also contribute to NBL. Among the different genes located in 1p36 locus, ARID1A, CASZ1 and RUNX3 are promising candidates due to their function as component of chromatin remodeling complexes (ARID1A) or known tumor suppressors (CASZ1 and RUNX3). Using shRNA approaches and CRISPR-CAS9 technology we are going introduced silencing mutations in the various gene loci to downregulate the expression of ARID1A and RUNX3 partially emulating the situation in 1p36 LOH which occur in NBL. Different guides RNAs (gRNAs) were designed to knockdown ARID1A, CASZ1 and RUNX3 expression. These gRNAs were tested separately to check their efficiency by genomic PCR, western blot and immunofluorescence (Data not shown). Then they were assembled all in one vector (VECTOR B) (Figure 9) in order to downregulate the expression of these three genes at the same time. The analysis shows a decrease of ARID1A (Fig.4) and RUNX3 (Fig.5-6) expression levels after transfection of VECTOR B in NCC lines. To emulate 1p36 LOH we are going to add the following candidates to our initial approach: CHD5, CAMTA1, miR34a and KIF1b. This is to help us decipher their potential role as oncogenic driver of each one of these genes.

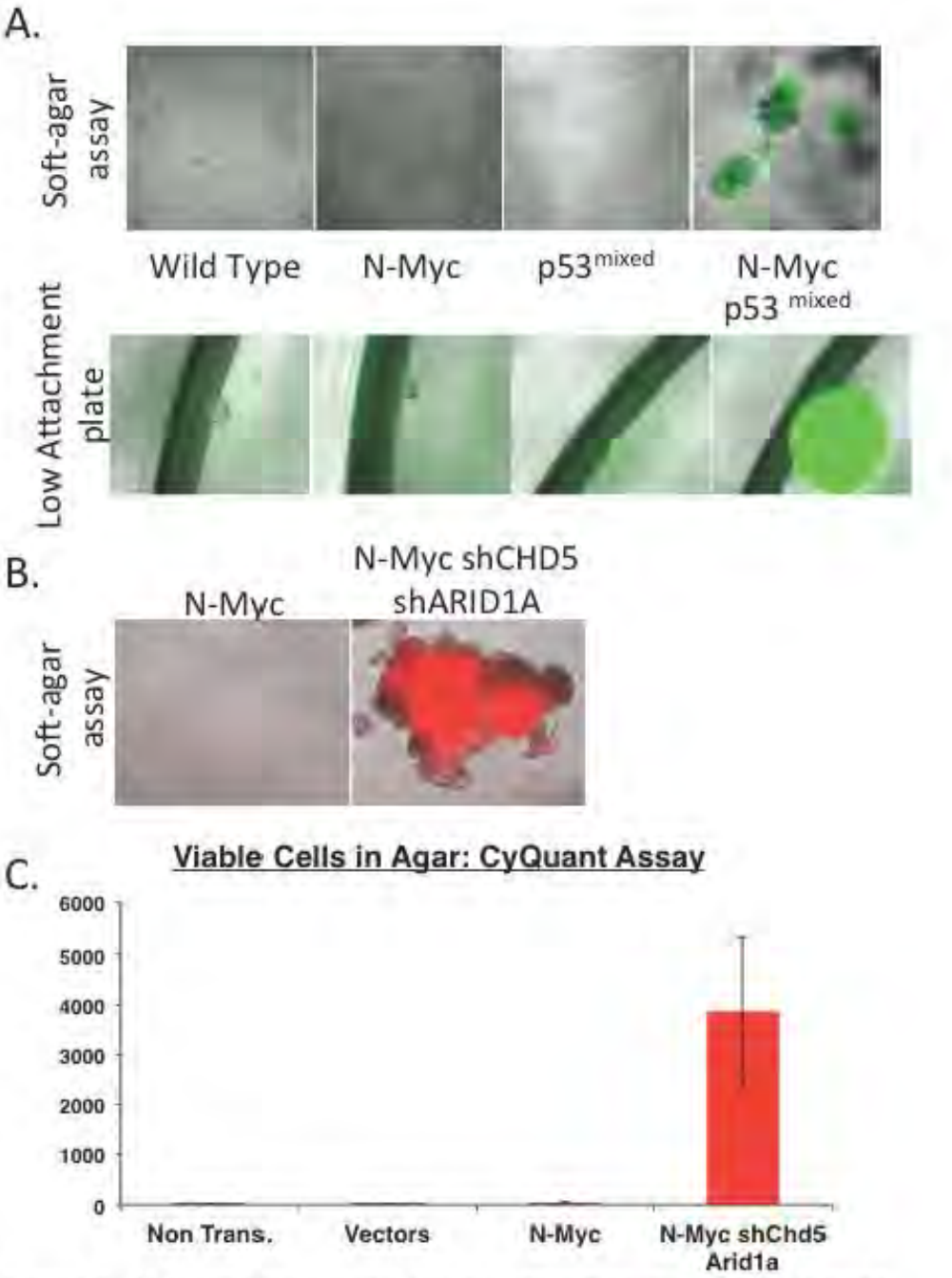

Figure 8: Soft-agar assays and colony formation in low-attachment plates are promising transforming screening approaches for primary NCCs to complement in vivo tumor formation genetic screens. N-Myc overexpresson in p53 compromised primary NCC leads to numerous (A) soft-agar colonies and (B) low attachment colonies while N-Myc alone is insufficient. B) Oevrexpression of N-Myc with both CHD5 and ARID1A silenced by shRNA in primary NCCs lead to robust soft agar colony formation as seen quantified in (C) by isolation of cells from the soft agar assay quantifed by CyQuant for live cell number.

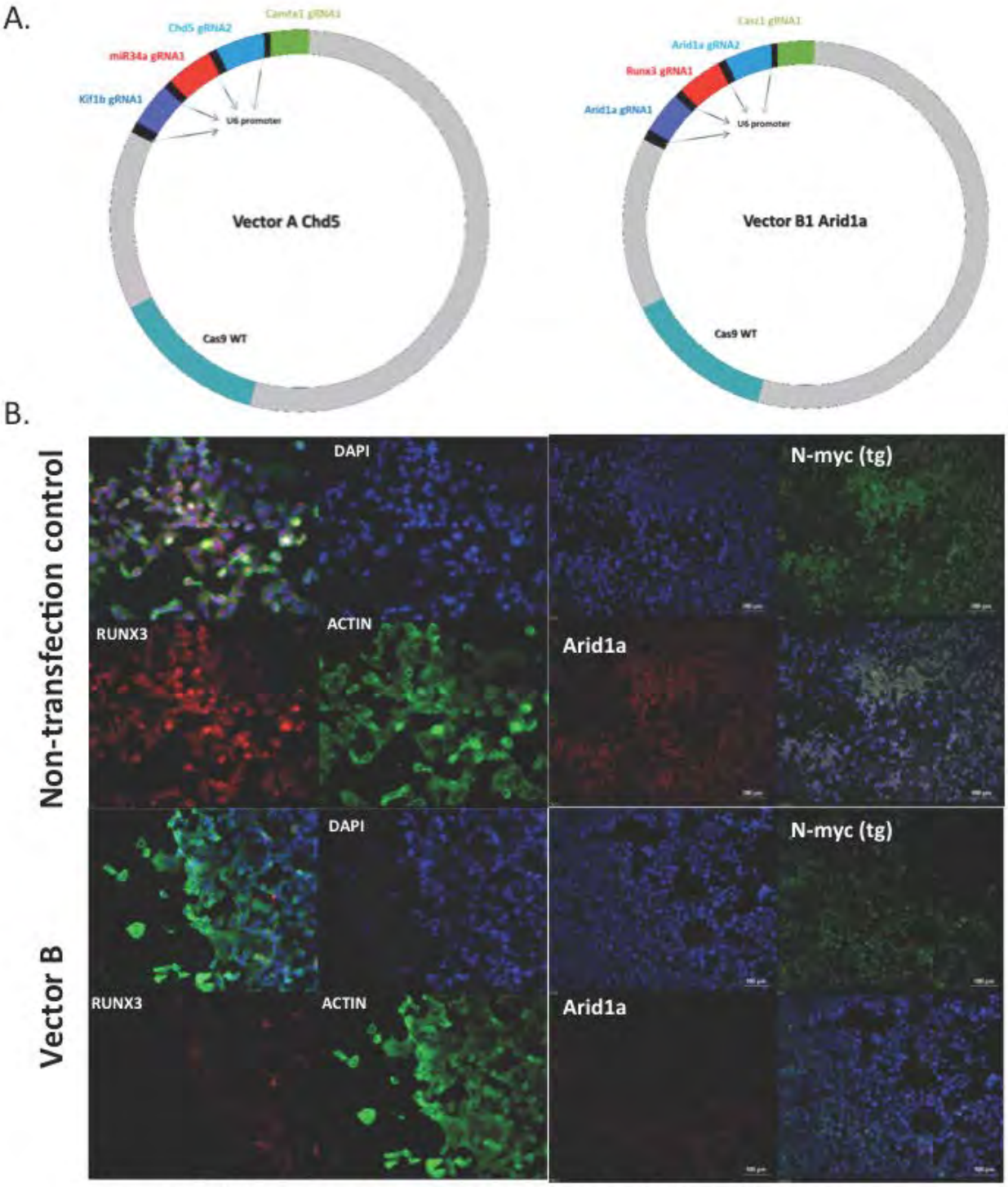

Figure 9: CRISPR-Cas9 vectors containing validated guide RNA (gRNA) to proposed neuroblastoma candidate genes located in the 1p36 locus. A) Plasmid diagrams showing arrangement of validated guide RNAs (gRNAs) to candidate genes in the 1p36 locus including mir34a, CHD5, CAMTA1, ARID1A, RUNX3 and CASZ1. B) Show examples of validated silencing of ARID1A and RUNX3 by immubofluorescence in cell line 72 hours after transfection of primary NCC with Vector B.

Subaim 2A; Creating lentiviral and retroviral library
30% completion
Subaim 2B; Pilot studies with NCC
70% completion
Subaim 2C: Screen of Pooled genes (shRNA and cDNA)
0% completion
Subaim 3C: Validation of identified candiates
0% completion

Training and professional development

Small group/ one-on-one meetings
As a mentored early career PI, in the past year I have had monthly meetings with Dr. Davidoff. I have also met with my career mentoring committee which includes- Dr. Michael Dyer, Dr. Brian Sorrentino, Dr. Douglas Greene and Dr. Kip Guy. I have also had biweekly meeting with other junior faculty under the guidance of the chair of the Genetics Department , Dr. Gerard Grosveld. I have also set up monthly meetings with the Cancer Center Director- Dr. Charles Roberts.

Collaborations
I have established collaborations in work related to but not directly involving this proposal with Dr. Kip Guy, Dr. Joseph Opferman and Dr. Scott Snyder.

Intra-institutional presentations
In the past year I have presented this work at the Developmental Biology and Solid Tumors cancer center program and the Cancer Genetics, Biochemistry and Cell Biology cancer center program. I have also presented at working groups focus on translational research for neuroblastoma and focused on epigenetics of cancer.

Results disseminated
Preliminary results were presented as poster presentations at two meetings
1) Keystone - Epigenetics and Cancer, January 25-30, 2015.
2) AACR- Annual Meeting Philadelphia, PA April 2015

Plan for next reporting period
We will finish reengineering our systems to utilize other promoters besides MSCV in order to better investigate the contribution of ARID1A to neuroblastoma. Though this will require generating new human neuroblastoma cell lines with silencing of ARID1A in our already established Myc-N inducible systems once this is done we can precede to repeat the analysis we outlined in the first aim, but with a defective system. We already have U6 driven shRNA that look effective (Figure 5). We are also subcloning N-Myc downstream of other promoters so we can take advantage of our floxed ARID1A mice and silencing of ARID1A in primary neural crest cells. Additionally we have a verified culturing system that now will allow us to start screening for oncogenic drivers. We are nearly completed on generating CRISPR-Cas9 guide RNAs that target the 1p36 locus and then will generate similar system for 11q LOH and use guide RNAs for an overexpression system that targets the 17q

locus. Any identified oncogenic drivers will be validated in both in vitro and in vivo systems that we have created and validated in the past year.

IMPACT:
Nothing to report

CHANGES/PROBLEMS:

Changes in approach: Nothing to report.

Actual or anticipated problems:
The following section is also in the accomplishment portion of the report above and the corresponding figures can also be found there.
We based our expression of N-Myc transgenes and shRNA constructs using MSCV promoters. We did this because they drove strong expression in neural tissue including neuroblastoma cell lines and in primary neural crest cells. We have determined that silencing of ARID1A either by using shRNA (Figure 4A and B) or CRISPR-Cas9 technology (Data not shown) leads to silencing of expression of N-Myc or red fluorescent protein (RFP) driven by an IRES in our constructs. We also speculate that there is a feedback loop leading to suppression of the shRNA itself as they are also driven off the MSCV promoter. Interestingly silencing of CHD5 in conjunction with silencing of ARID1A restores expression from the MSCV promoter (Figure 4A and B) showing a functional interaction of these two putative tumor suppressors from the 1p36 locus but also further confounding some of the results that we have generated to this point. This also explains issues that were arising in our inducible cell line (iN-Myc-AS and iN-Mys-FI) where we saw changing levels of ARID1A knockdown when we try to look at expression of the gene during timecourse studies, proliferation studies and apoptosis studies. This necessitates us to rebuilding these systems ,which we are currently doing (data not shown).

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Changes that had a significant impact on expenditures:
Nothing to report

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents:
Nothing to report

PRODUCTS:

Journal publications.
Nothing to report

Books or other non-periodical, one-time publications.
Nothing to report

Other publications, conference papers, and presentations:

1) A Primary Cell-Based Assay to Identify Initiating Oncogenic Events in Neuroblastoma
Rachelle R. Olsen, Zhirong Yin, **Kevin W. Freeman**
Keystone - Epigenetics and Cancer, January 25-30, 2015. *Selected for poster presentation.*

2) A Primary Cell-Based Assay to Identify Initiating Oncogenic Events in Neuroblastoma
Rachelle R. Olsen, Zhirong Yin, **Kevin W. Freeman**
AACR- Annual Meeting Philadelphia, PA April 2015. *Selected for poster presentation.*

Website(s) or other Internet site(s)
Nothing to report

Technologies or techniques
We have developed assays both in vivo and in vitro using primary mouse neural crest cells for functionally screening candidate oncogenic drivers of neuroblastoma.

Inventions, patent applications, and/or licenses
Nothing to report

Other Products
Nothing to report

PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

Individuals who have worked on the project:

Name: Kevin Freeman *Project Role:* PI *Nearest person month worked:* 7 months
Contribution to Project: Experimental design, mentoring researchers, troubleshooting experiments *Funding Source*: CDMRP grant and institutional funds

Name: Rachelle Olsen *Project Role:* Postdoctoral Fellow *Nearest person month worked:* 11 months
Contribution to Project: Experimental design, performing experiments
Funding Source: CDMRP grant and institutional funds

Name: Joel Otero *Project Role:* Senior Research Technician *Nearest person month worked:* 10 months

Contribution to Project: Experimental design, performing experiments
Funding Source: CDMRP grant and institutional funds

Name: Jesus Garcia-Lopez *Project Role:* Post-doctoral fellow *Nearest person month worked:* 5 months
Contribution to Project: Experimental design, performing experiments
Funding Source: Institutional funds

Name: Zhirong Yin *Project Role:* Senior Research Technician *Nearest person month worked:* 4 months
Contribution to Project: Experimental design, performing experiments
Funding Source: Institutional funds

Name: Kirby Wallace *Project Role:* Graduate Student *Nearest person month worked:* 3 months
Contribution to Project: Experimental design, performing experiments
Funding Source: University of Tennessee Graduate School and CDMRP grant

Changes in key personnel

Zhirong Yin left her position as a senior research technician. Joel Otero was moved from being funded from the CDMRP grant to the vacated institutional position. Kirby Wallace will be funded from the CDMRP grant.

Other Organizations
Nothing to report

SPECIAL REPORTING REQUIREMENTS

None, nothing to report